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ARTIFICIAL INDUCTION OF OOCYTE MATURATION AND DEVELOPMENT IN THE SEA CUCUMBERS *HOLOTHURIA LEUCOSPILOTA* AND *HOLOTHURIA PARDALIS*[1]

YOSHIHIKO K. MARUYAMA

Department of Zoology, Faculty of Science, Kyoto University, Kyoto 606, Japan

Although a number of works have been written on the development of holothurians (MacBride, 1914; Korschelt, 1936; Hyman, 1955; Inaba, 1957; Oguro, 1974), extensive experimental studies on development have not been carried out. One reason for this may be difficulties in obtaining mature eggs: selection of mature eggs from excised ovaries or induction of spawning has been practical for only some species of holothurians (Ohshima, 1925a, b; Colwin, 1948; Inaba, 1957; Strathman and Sato, 1968). In two species common on the coast of Japan, *Holothuria leucospilota* and *Holothuria pardalis*, mature eggs are rarely found in excised ovaries and oocytes isolated from ovaries do not mature spontaneously in sea water.

The present paper reports success of artificial maturation of isolated oocytes from ovaries of *H. leucospilota* and *H. pardalis*. With the use of some disulfide reducing agents, nearly all full-grown oocytes of these two species were induced to mature. These mature oocytes formed the first and second polar bodies, and after insemination they developed to typical auricularia larvae.

Materials and Methods

Specimens of *Holothuria leucospilota* and *Holothuria pardalis* were collected during a presumed breeding season (July to August) at the coast near the Seto Marine Biological Laboratory of Kyoto University.

Isolated ovaries were washed with sea water three times. Oocytes excised from the ovaries with fine forceps were further washed with sea water three times, and then used for experiments. These isolated oocytes rarely matured spontaneously in sea water. During this season, the oocytes were rather uniform in size. Oocytes of *H. leucospilota* were transparent and pale pink in color and those of *H. pardalis* were transparent and colorless.

Dithiothreitol (DTT) (Wako Pure Chemical Industries and Nakarai Chemicals, Kyoto), 2,3-dimercapto-1-propanol (BAL) (Wako), L-cysteine (Sigma Chemical Co., St. Louis) and glutathione (reduced form) (Wako) were dissolved in sea water just before use. 1-methyladenine (1-MA) (Sigma) also prepared for experiments of oocyte maturation, was dissolved in distilled water at 1×10^{-3} M and diluted with sea water just before use (Kishimoto and Kanatani, 1973).

The subsequent experiments were carried out at the water temperature of 28–29° C.

Results

Induction of oocyte maturation with dithiothreitol (DTT)

The DTT solution (1×10^{-3} M in sea water) induced the breakdown of germinal vesicles of immature oocytes from *H. leucospilota* and *H. pardalis*.

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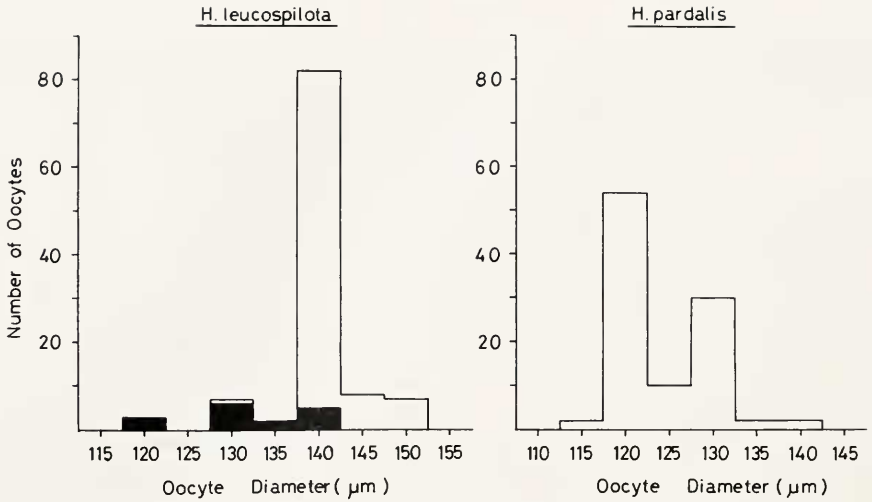

FIGURE 1. Diameter distribution of mature and immature oocytes after DTT treatment (1×10^{-3} M). Open and shaded columns indicate mature and immature oocytes, respectively. Average diameter of mature oocytes: in *Holothuria leucospilota* 141 ± 4 μm (means ± s.d., n = 100) and in *Holothuria pardalis* 124 ± 5 μm (means ± s.d., n = 100). In this sample of *H. pardalis*, all oocytes were induced to mature.

In the case of *H. leucospilota,* however, germinal-vesicle breakdown was not observed in small oocytes (less than 130 μm in diameter), which suggests that these oocytes are not full-grown. Only those oocytes exceeding 130 μm in diameter were studied, on the assumption that these alone are full-grown. Small oocytes (under 130 μm) were not scored.

In *H. pardalis,* only oocytes with a diameter of more than 115 μm were regarded as full-grown.

The diameter distributions of the oocytes are shown in Figure 1.

DTT treatment necessary for germinal-vesicle breakdown

A dose-response curve of oocyte maturation in *H. leucospilota* with various concentrations of DTT solution (1×10^{-5}–1×10^{-1} M) is shown in Figure 2A. Almost 100% of oocytes matured in 5×10^{-4}–1×10^{-2} M DTT solution. At concentrations beyond this range, oocytes rarely matured.

The duration of DTT treatment (1×10^{-3} M) required for induction of oocyte maturation in *H. leucospilota* was determined (Fig. 2D). The curve of germinal-vesicle breakdown *versus* the period of treatment shows that a 10-min treatment induces such breakdown in almost all oocytes and that a threshold exists at 3–5 min in *H. leucospilota* and at 10 min in *H. pardalis* (not shown), at 28–29° C. Nearly all oocytes induced to mature by DTT developed when inseminated (see later sections).

The effects of other disulfide reducing agents and 1-MA were also tested. BAL (5×10^{-3} M) and L-cysteine (5×10^{-2}–1×10^{-1} M) successfully induced 100% of oocyte maturation (Figs. 2B and 2C), although a very high concentration was required in the case of L-cysteine. The process and time course of maturation by these agents were almost the same as those by DTT.

1-MA (1×10^{-6}–1×10^{-4} M) and glutathione (reduced form, 1×10^{-6}–3×10^{-1} M) failed to induce oocyte maturation in *H. leucospilota*.

From these observations, a 10-min treatment with 1×10^{-3} M DTT solution was chosen as the standard procedure for maturation. The treated oocytes were washed with sea water three times to remove DTT and then used as materials in the following experiments.

Process of oocyte maturation

Since holothurian oocytes are spawned at the metaphase of the first meiotic division under natural conditions (Ohshima, 1925a, b; Costello *et al.*, 1957), the earlier processes of maturation have been very difficult to study. Here the process of germinal-vesicle breakdown and meiotic division will be described in some detail, mainly as it occurs in oocytes of *H. leucospilota*.

A large germinal vesicle is situated in the center of each oocyte, and one large nucleolus is observed within it (Fig. 3A). From the oocyte surface numerous radial fibers extend through a transparent jelly layer to a follicle coat (Figs. 3A-3H). A single transparent cytoplasmic process (termed the micropyle process) (Ohshima, 1925a, b; Lindahl, 1932) extends from the oocyte surface through the jelly layer and its tip attaches to the follicle coat (Fig. 3A). Isolated oocytes do not always possess follicle coats; some have been already stripped off (Fig. 3B).

About 10–15 min after the start of DTT treatment, oocytes lose follicle coats, which form clumps near the micropyle process (Fig. 3B) or sometimes near the antipole of the process. In those oocytes which have been stripped of follicle coats, the tip of the process becomes rounded (Fig. 3C). The shape of the process is maintained until the first meiotic division. Jelly coat (20 μm thick) is not removed by DTT treatment.

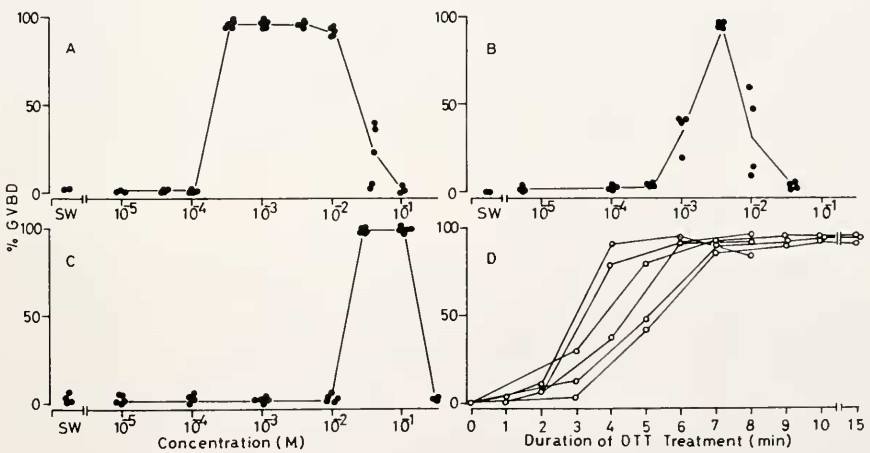

FIGURE 2. Effects of concentrations of DTT (A), BAL (B) and L-cysteine (C) and durations of DTT treatment (1×10^{-3} M) (D) on oocyte maturation in *Holothuria leucospilota*. Each point represents the reponse of 100–180 oocytes. Parts A, B and C show the rates of matured oocytes counted 90–120 mm after the start of treatment with these agents. SW indicates the control oocytes in sea water. In part D, oocytes treated with DTT for various durations were washed with sea water three times and placed into sea water. The rates of germinal-vesicle breakdown (GVBD) were counted 30–50 min after the start of DTT treatment.

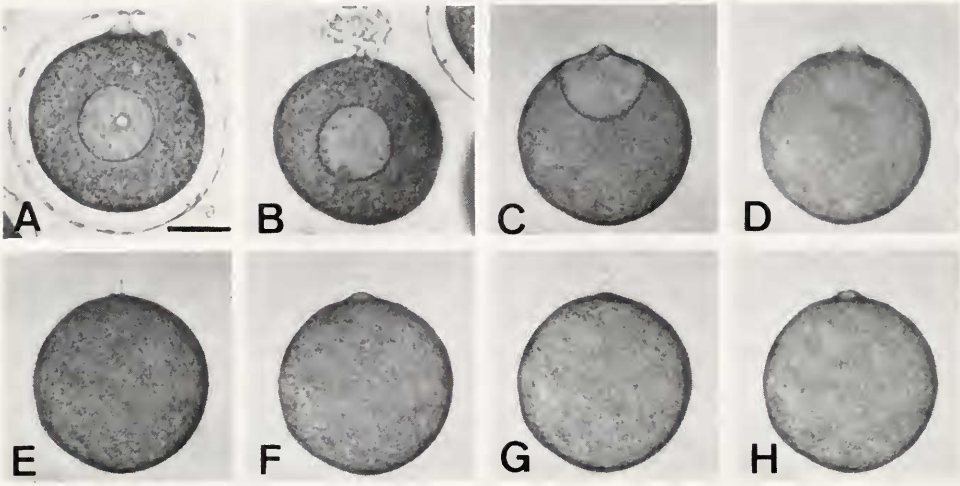

FIGURE 3. Process of oocyte maturation induced by DTT treatment (*Holothuria leucospilota*); (A) and (B) Isolated oocytes, (C) 19 min after the start of DTT treatment, (D) 24 min, (E) 55 min, (F) 65 min, (G) 80 min, (H) 100 min. All pictures are at the same magnification (bar: 50 μm).

At 14 min after the start of DTT treatment, the germinal vesicle begins moving to the site of the micropyle process and at 18 min, assumes the shape of a compressed hemispheroid with one side apparently attached to the inner surface of the oocyte membrane at the site of the micropyle process (Fig. 3C). At 20 min, the nuclear envelope suddenly disintegrates (Fig. 3D). Formation of the first polar body (at 55–60 min) is initiated by contraction of the surface near the micropyle process and subsequent conspicuous elongation of the process (Fig. 3E) and is completed with a pinching-off near its base. Soon after this, a small bulge is formed at the site of the micropyle process (Fig. 3F). At 70–80 min, the second polar body is pinched off from the same place (Figs. 3G and 3H) in the same fashion as the first polar body. The maturation process of *H. pardalis* is almost the same as that of *H. leucospilota*.

Fertilization and the first cleavage

The first discernible reaction of oocytes after insemination is formation of the fertilization cone and wrinkling of the oocyte surface. A few minutes later, the fertilization membrane is formed, although its height is low (Fig. 5A). Immature oocytes, when inseminated, protrude many cytoplasmic processes (probably fertilization cones), which suggests polyspermy. They do not mature or develop.

The spatial relationship between the polar bodies and the fertilization membrane is diverse: insemination before the first meiotic division is followed by formation of both the first and second polar bodies on the oocyte surface within the perivitelline space. When fertilized between the first and second meiotic divisions, oocytes elevate the first polar body with the fertilization membrane and form the second one on the oocyte surface within the perivitelline space. Fertilization after the second meiotic division results in elevation of both polar bodies with the fertilization membrane.

Mature oocytes were inseminated at various stages of the maturation process in order to determine the timing of fertilization appropriate for further development (Fig. 4A). Monospermic fertilization was possible after germinal-vesicle breakdown (20 min). When inseminated thereafter, until 60 min after the start

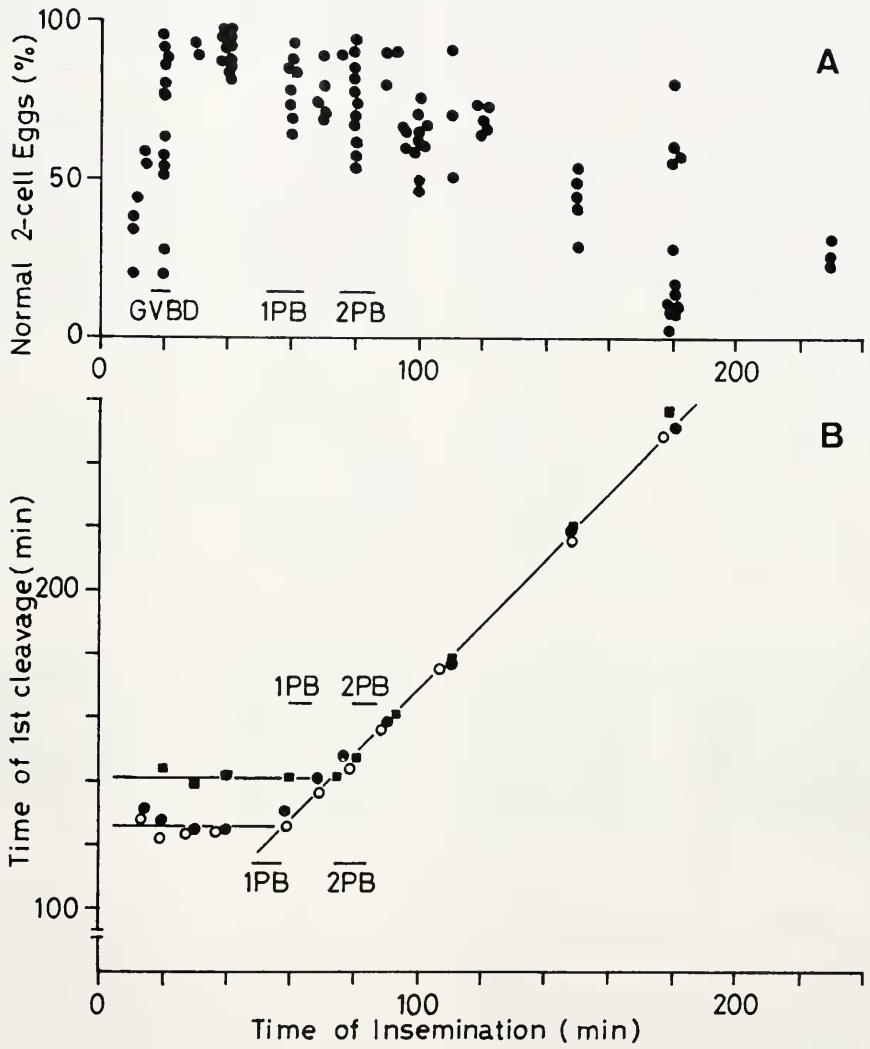

FIGURE 4. Correlations between the time of insemination and the frequency of normally dividing eggs (A), and between the time of insemination and initiation of the first cleavage (B) (*Holothuria leucospilota*). The time of insemination and initiation of the first cleavage was clocked from the start of DTT treatment. The frequency of the dividing eggs was counted with 5-min intervals, and the time when 50% of the eggs divided was referred to as the time of the first cleavage. 1PB and 2PB show the first and the second polar-body formation respectively. In part B, upper signs (1PB and 2PB) are for one experiment from one batch (shaded boxes) and lower signs (1PB and 2PB) are for two experiments from the other batch (open circles and closed circles). The right portion of the curves is the linear regression curve to the plots after the time of formation of the first polar body in these three experiments ($Y = 1.0X + 64.0$, $r = 1.00$). Each point (in A and B)represents the response of 70–230 eggs.

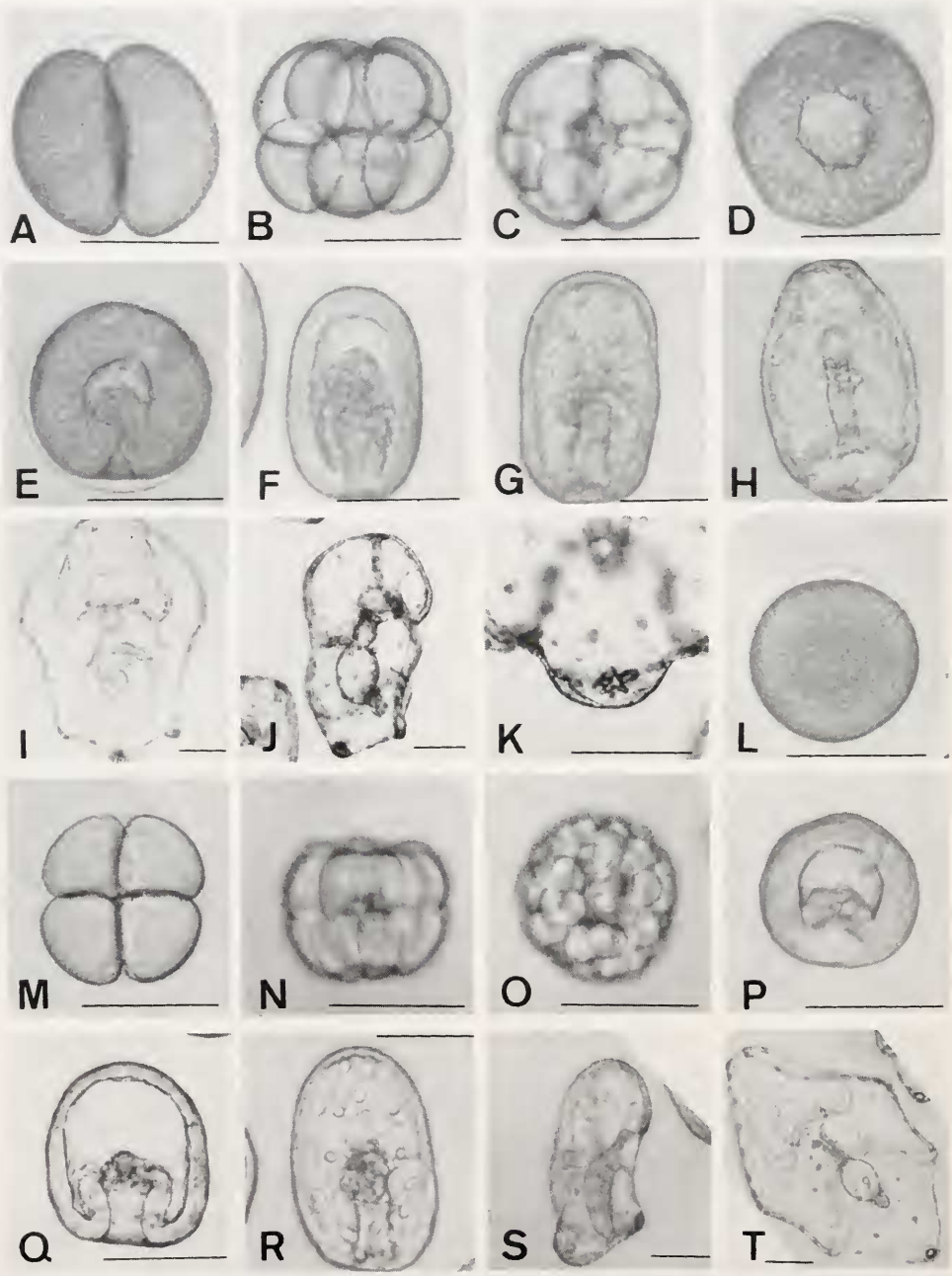

FIGURE 5. Early development of *Holothuria leucospilota* (A-K) and *Holothuria pardalis* (L-T); (A) 2-cell egg (1 hr 30 min after insemination), (B) 8-cell egg (2 hr 40 min), (C) 16-cell egg (3 hr 25 min), (D) Blastula (10 hr 40 min), (E) Early gastrula (13 hr) with invaginating archenteron (just before hatching), (F) Middle gastrula (19 hr), (G) Late gastrula (27 hr) with migrating mesenchymal cells, (H) Early auricularia (39 hr, ventral view), (I) Auricularia (140 hr, ventral view), (J) Auricularia (74 hr, side view), (K) The rear end of 70 hr auricularia, (L) 1-cell egg with two polar bodies, (M) 4-cell egg, (N)

of DTT treatment, 90–100% of mature oocytes divided normally at the first cleavage (later, almost all these oocytes developed to gastrulae). The frequency of normally dividing eggs decreased as insemination was delayed further: The frequency was 30% when they were inseminated at 230 min. The suitable timing of insemination would be, therefore, the period between the completion of germinal-vesicle breakdown and meiotic division.

The interval between insemination and first cleavage was found to depend on the state of maturation of the egg (Fig. 4B). The time of the first cleavage is represented by the time when 50% of the eggs had divided. When oocytes were inseminated at various times before first polar-body formation, they began the first cleavage at the same moment within the same batch (approximately 120–140 min after DTT treatment). Inseminated oocytes formed both polar bodies almost simultaneously with unfertilized mature oocytes. When oocytes were inseminated after the first meiotic division, the interval between DTT treatment and cleavage increased. However, the slope of the linear regression curve is 1.0 (correlation coefficient: $r = 1.00$), which means that the interval from insemination to the first cleavage is constant (about 64 min), and presumably dependent upon the time of fertilization. These results indicate that 1) the process of maturation including egg fertilizability is set prior to and independently of fertilization, 2) the process for the first cleavage could be initiated after the first meiotic division, and 3) after that division, fertilization immediately triggers the processes that result in cleavage.

Development of fertilized eggs

Mature eggs of *H. leucospilota*, inseminated before the first meiotic division (40 min after the start of DTT treatment) were used for observation of further development. At 90 min after fertilization, the first cleavage furrow passed through the animal-vegetal axis (Fig. 5A). About 35 min later, the second cleavage followed the first one with the cleavage plane perpendicular to that of the first division. The third (Fig. 5B) and the fourth cleavages, planes of which were equatorial and meridional respectively, gave rise ultimately to 16 equal blastomeres (Fig. 5C). Up to the 7th cleavage each blastomere divided every 35–45 min, rather synchronously. After the 7th cleavage the divisions became asynchronous and the intervals were prolonged. The surface of the blastula was not wrinkled (Fig. 5D). About 12 hr after fertilization the embryos began to spin within the fertilization membrane. At almost the same time, invagination at the vegetal pole was also initiated (Fig. 5E). The direction of spin of embryos within the fertilization membrane was either clockwise or counter-clockwise as viewed from the vegetal pole. Embryos hatched from the fertilization membrane 13 hr after fertilization. After hatching, all embryos spun counter-clockwise around the animal-vegetal axis as viewed from the vegetal pole, and swam with the animal pole leading, in the same fashion as sea-urchin embryos (Maruyama, unpublished). As gastrulation proceeded, mesenchymal cells began migrating from the archenteron tip into the blastocoel (Fig. 5G). At about 27 hr, the stomodeum at the ventral surface was attached by the bent tip of the archenteron, and cells at

16-cell egg, (O) 64-cell egg, (P) Early gastrula (13 hr) with invaginating archenteron (just before hatching), (Q) Middle gastrula (14 hr), (R) Late gastrula (23 hr) with migrating mesenchymal cells, (S) Early auricularia (39 hr, side view) with the ciliary ridge and the pore canal, (T) Auricularia (93 hr). Bars indicate 100 μm.

the tip attached to the median dorsal gastrula wall and formed the dorsal pore. At 39 hr, the larvae were at the early auricularia stage (Fig. 5H). The ciliary ridges of the larvae became clear. The position of the anus shifted to the ventral side. At the rear of the body a small birefringent granule appeared. This is probably a calcareous structure, because the birefringence was so strong as to be detectable by a pair of sheets of plastic polaroid. The position of the dorsal pore shifted slightly to the left side as viewed from the dorsal surface (Fig. 5H). At about 46 hr, the ciliary ridge became more and more clear. In side view, a tubular pore canal connected the dorsal pore with the coelom at the position of the oesophagus. At 70 hr, the larvae developed to the typical auricularia (Figs. 5I and 5J). The conspicuous ciliary ridge, which consisted of a single continuous loop, was formed (Figs. 5I and 5J). The birefringent body was observed only in the center of the rear of the larva and now grew to a characteristic four- or five-armed shape (Fig. 5K). The gut was divided into three parts: oesophagus, stomach and intestine. Rhythmic contraction and relaxation of the stomach began. These larvae swam with the head forward and spun counter-clockwise around the long axis of the larval body, as viewed from the rear of the larva.

Development of *H. pardalis* (Figs. 5L-5T) was similar to that of *H. leucospilota,* except that the mature birefringent structure was globular in shape (Fig. 5T).

Discussion

Artificial maturation of full-grown oocytes with BAL, DTT and L-cysteine seems to be, at present, the only useful method for easily obtaining mature eggs of sea cucumbers. The standard procedure presented in this paper was also applicable to immature oocytes of *Holothuria pervicax* and *Holothuria moebi* (Maruyama, unpublished). Oocytes thus matured could be fertilized and developed to the auricularia stage, with the developmental process very similar to that in other species of sea cucumbers reported previously (MacBride, 1914; Korschelt, 1936; Hyman, 1955; Inaba, 1957; Oguro, 1974). This indicates that the eggs obtained in this way are essentially normal.

The cleavage pattern was total-equal, as in the well-known case of cleavage of *Synapta digitata* described by Selenka (MacBride, 1914). About 2 days after fertilization, the ciliary ridge was formed as a single continuous loop, which characterizes the auricularia larva of the sea cucumber (MacBride, 1914; Korschelt, 1936; Hyman, 1955; Inaba, 1957; Oguro, 1974). The birefringent calcareous body, the shape of which was species-specific in each of the two species studied, was formed at the rear of the larval body. Müller and Mortensen described this body as the larval spicule in auricularia larvae of *Holothuria nigra, Holothuria tubulosa* and others whose adult forms are unknown (Mortensen, 1913; Ohshima, 1911).

1-MA (known as the oocyte maturation hormone in starfishes; Kanatani *et al.,* 1969) failed to induce oocyte maturation of sea cucumbers. Identification of a natural maturation hormone specific to the sea cucumber is awaited. At any rate, maturation of oocytes with disulfide reducing agents in sea cucumbers and starfishes indicates that the biochemical mechanisms underlying their processes of maturation probably are similar: The reduction of disulfide bonds may play an important role in the oocyte maturation of both (Kishimoto and Kanatani, 1973; Kishimoto *et al.,* 1976). In the DTT-matured eggs, fertilization-membrane eleva-

tion was low. DTT has been believed to have some action destructive to the vitelline envelope, as has been reported in sea urchins (Epel *et al.*, 1970; Vacquier *et al.*, 1972), starfishes (Kishimoto and Kanatani, 1973; Kishmoto *et al.*, 1976) and sea lilies (Holland, 1976).

One significant feature in holothurian oocytes is the existence of the micropyle process, which has attracted attention because it might have a causal relationship with the site of polar body extrusion (Ohshima, 1925a, b). Results reported in this paper indicate that 1) the germinal vesicle migrates to the micropyle process and disappears there, 2) the first and second polar bodies are extruded from the process and, 3) the first cleavage furrow passes near the site of polar bodies. These observations favor the hypothesis that the micropyle process is a preformed structure, laid down during oogenesis, which participates in meiosis and determination of egg polarity. Thus, the micropyle process might itself manifest the "animal pole."

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Summary

Isolated oocytes of the sea cucumbers *Holothuria leucospilota* and *Holothuria pardalis* were induced to mature with 2,3-dimercapto-1-propanol (BAL), dithiothreitol (DTT) and L-cysteine. 1-Methyladenine, known as the maturation-inducing hormone of starfishes, failed to induce oocyte maturation of the sea cucumbers. Observation of the maturation process revealed that the germinal-vesicle of the oocyte treated with DTT migrated to the micropyle process and broke down there, where polar bodies were subsequently formed. After insemination, mature eggs obtained with DTT treatment developed to the typical auricularia larvae.

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